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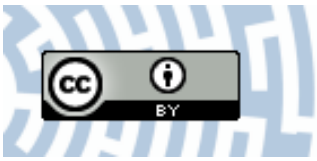

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Thin-layer chromatographic quantification of magnolol and honokiol in dietary supplements and selected biological properties of these preparations

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ABSTRACT

Two isomeric biphenyl neolignans, magnolol and honokiol, are considered as constituents responsible for the healing effect of magnolia bark, a traditional Oriental medicine. To survey the increasing number of dietary supplements that contain magnolia bark or its extract, an affordable quantitative thin-layer chromatography (TLC) – densitometry method was developed. The methanol extracts were analyzed on the silica gel plates after manual sample application using *n*-hexane – ethyl acetate – ethanol (16:3:1, v/v/v) as a mobile phase. For quantitation, the chromatograms were scanned in the absorbance mode at the wavelength $\lambda = 290$ nm. The limits of detection and quantitation were 90 and 280 ng/zone for magnolol and 70 and 200 ng/zone for honokiol, respectively. None of the two targeted neolignans were detected in two of the six analyzed supplements. In the other four samples, the measured amounts were between 0.95-114.69 mg g^{-1} for magnolol and 4.88-84.86 mg g^{-1} for honokiol. Moreover, separations of these two neolignans on the TLC and high-performance TLC (HPTLC) layers were compared and HPTLC was combined with antioxidant (DPPH) and antibacterial (*Bacillus subtilis* and *Aliivibrio fischeri*) assays and mass spectrometry (MS), using the elution-based interface. Both magnolol and honokiol exhibited effects in all bioactivity assays. The HPTLC-MS tests confirmed purity of neolignan zones in the extracts of dietary supplements and supported tentative identification of the alkaloid piperine and the isoflavone daidzein as additional bioactive components of the investigated dietary supplements. Using the same mobile phase in the orthogonal directions 2D-HPTLC-MS experiments proved degradation, i.e., instability of magnolol and honokiol on the silica gel adsorbent.

1. Introduction

Concentrated sources of nutrients (i.e., vitamins and minerals) and other compounds with a nutritional or physiological effect (e.g., different amino acids, fatty acids, fibers of herbal origin, and various plant and herbal extracts) marketed in a "dose" form (e.g., as pills, tablets, capsules, or liquids in measured doses) are regarded as dietary supplements. According to the EU General Food Law Regulation (EC) No 178/2002 [1], dietary supplements are considered as foodstuffs and therefore they are regulated as foods. The guidelines for handling and control of dietary supplements (other than vitamins and minerals) are provided by the European Food Safety Authority (EFSA) in the form of regulations. Special importance is attributed to the safety of ingredients used in dietary supplements of botanical origin [2] and to defining the highest levels of chronic daily intake of a nutrient that is not likely to pose a risk of adverse health effects to humans. Observations of the global market of dietary supplements prove that it expands vigorously, and at the same time, that respective legal regulations are less strict than those regarding pharmaceuticals. Consequently, there is an acute need for accurate, robust and cost-friendly analytical methods to control individual components of the dietary supplement formulations.

With growing awareness in the West of the considerable healing potential of the traditional medicines of the Far East Asian countries, herbal preparations and individual compounds isolated from Oriental plant material are introduced into various different

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dietary supplements (and also into cosmetics) as active components. Two isomeric biphenyl neolignans, magnolol and honokiol, obtained from the bark of different magnolia species, are gaining popularity as active components of dietary supplements, with two representatives of the Magnoliaceae family, i.e., *Magnolia officinalis* Rehder & E.H. Wilson (in Chinese Hou-po) and *Magnolia obovata* Thunb, being most often used for this purpose [3]. Bark and flowers of the magnolia species, either alone, or in combination with other herbal preparations, have been present in traditional Chinese, Japanese and Korean medicines for thousands of years, basically as remedies for gastrointestinal disorders, anxiety and allergies [4, 5], but also for their sedative, antioxidant, anti-inflammatory, antibiotic, and antispastic effects [6]. It is generally accepted that magnolol and honokiol are the most active compounds of the magnolia bark extracts (often referred to as MBE), and for this reason, biological and pharmacological investigations of MBE most often focus on these two neolignans [5, 7-9]. Toxicological studies performed for different quantitative compositions of MBE using different animal models confirmed its safety for oral consumption. Moreover, a no-observable-effect level (NOEL) for the concentrated MBE was established at > 240 mg MBE per kg body weight per day, as an oral dose [10].

Magnolol and honokiol exert a well documented antioxidant activity. The most complex physicochemical study focusing on the kinetics and mechanism of the reaction of magnolol and honokiol with peroxyl radicals has been presented in a paper [11]. Antioxidant activity of these two neolignans was compared with that of the other structurally related phenolics and the analyses suggested the role of allyl groups in the effect due to the ability to scavenge O_2^- or the hydroxyl radical [12]. Antidiabetic and antioxidant activity of honokiol was proven *in vivo* on rats with diabetes induced by the high-fat diet and streptozotocin [13]. Magnolol and honokiol additionally displayed significant antifungal activity against various human pathogenic fungi, e.g. *Aspergillus niger, Cryptococcus neoformans*, and *Candida albicans* [14], and antibacterial effect against e.g. *Staphylococcus aureus*, MRSA [15], *Propionibacterium acnes* and *P. granulosum* [16], *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Micrococcus luteus*, and *Bacillus subtilis* [17]. In a review paper [18], providing 84 references to the original research publications, an abundant overview was made of the biological and physiological activities of magnolol.

Initially, thin-layer or column chromatographic separation of the structurally similar magnolol and honokiol has been quite difficult [19-21]. The first report on the determination of these two neolignans, with the aid of high-performance liquid chromatography with photodiode array detection (HPLC-DAD), was published in [22], and a new HPLC-DAD method to quantify magnolol in modern pharmaceutical formulations is given in [23]. Separation of magnolol from its metabolites by means of liquid chromatography-mass spectrometry (LC-MS) was reported in [24] and separation and quantification of magnolol and honokiol contained in *Magnoliae officinalis* cortex by means of high-performance thin-layer chromatography (HPTLC) was presented in [25]. The authors of the latter study quantified honokiol and magnolol in the cortex samples, exploiting their antioxidant effects with use of an HPTLC-DPPH· (2,2-diphenyl-1-picrylhydrazyl radical) assay followed by densitometric evaluation. Using of DPPH· as a visualizing agent is not recommended though, in view of an easily available direct detection and quantification method by means of densitometry.

In paper [26], the authors also used TLC to analyze magnolol and honokiol extracted from the *Magnolia officinalis* cortex, but again they did not evaluate their chromatograms by simple densitometry. Instead, they visualized the chromatograms by spraying them with the vanillin reagent followed by heating. Several reports on applications of TLC to the analysis of magnolol and honokiol from the different botanical sources are available in Chinese [27-33] and for this reason, detailed procedures and discussion of the results obtained practically escape Western readers, except for raw data regarding stationary and mobile phase, and the retention parameter values. An additional drawback is that six out of these seven papers originate from the nineties of the past century and three of them [30-32] are almost thirty years old. Although in each case silica gel was reported as stationary phase, without an access to full contents of these papers one cannot be sure, if the authors have worked with self-precoated chromatographic plates, or with the commercial ones. In this sense, the results reported in papers [27-33] are practically irreproducible and therefore of lesser importance. Furthermore, in most of these methods the mobile phase contains benzene [27-28, 30-33], which is carcinogenic and therefore not advised to use.

On the other hand, (HP)TLC is an affordable technique enabling a parallel analysis of a higher number of samples, without an expensive and complex sample preparation [34]. Moreover, as an open system, it is easily combined with bioactivity assays, providing the non-target screening and bio-profiling of the samples for bioactive components [35, 36].

On account of an increasing availability of commercial preparations which include magnolol and/or honokiol, and due to the lack of accurate, robust and cost-friendly methods to quantify these two neolignans, an effort was undertaken to develop a silica gel-based normal phase (NP) thin-layer chromatographic procedure that is well suited for the task. Moreover, the TLC method was transferred to HPTLC that was combined with rapid bioautographic screening (effect-directed analysis, EDA) for the antibacterial and antioxidant potential of the considered dietary supplements.

2. Materials and methods

2.1. Materials

All quantification studies were carried out with use of TLC silica gel 60 F_{254} plates (20 cm × 20 cm, #105715; Merck, Darmstadt, Germany). Otherwise TLC (#105554) and HPTLC (#105547) silica gel 60 F_{254} layers (Merck) were applied. Analytical grade solvents for (HP)TLC were from PPH POCh (Gliwice, Poland) or Molar Chemicals (Budapest, Hungary). Gradient grade methanol was purchased from Merck or Molar Chemicals. As phytochemical standards, magnolol and honokiol samples were used (>99 % purity; Selleck Chemicals, Houston, TX, USA). Eight samples of commercial products, containing MBE or pure magnolol and/or honokiol as declared by the manufacturers, were also used. Two samples were magnolol and honokiol mixtures in undeclared quantitative proportions, kindly donated as commercial raw materials for the preparation of dietary supplements and they were labeled as Mix 1 and Mix 2, respectively. The remaining six samples (presented in Table 1) were dietary supplements purchased from Polish and Hungarian online shops or pharmacies. The Gram negative, naturally luminescent marine bacterium *Aliivibrio fischeri* (DSM 7151) was obtained from the Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Cultures (Berlin, Germany) and the Gram positive soil bacterium *Bacillus subtilis* strain F1276 was kindly provided by József Farkas (Central Food Research Institute, Budapest, Hungary).

2.2. Preparation of standard solutions and extracts of dietary supplements

Solutions of the magnolol and honokiol standards were prepared in methanol at the concentration of 0.1 mg mL^{-1}, and stored in a refrigerator at + 6°C. Samples of Mix 1 and Mix 2 were dissolved in methanol at the concentration of 0.1 mg mL^{-1}. With the

Table 1
Basic characteristics of the six magnolol and/or honokiol containing dietary supplements (samples 1-6) (MBE: the magnolol bark extract as the source of the two investigated neolignans).

Sample no.	Source of main component(s)	No. of tablets/capsules/ liquid volume per one package	Components of interest detected by normal phase TLC (SiO_2)	Average mass/volume per one tablet/ capsule/vial (% RSD)	Declared quantity per one tablet/capsule/vial				Components with undeclared quantities
					MBE	Magnolol	Honokiol	Other components	
1	Magnolia bark extract	30 tablets	Magnolol, honokiol	604 mg (0.74)	6 %	No data	21 mg	Piperine, 5 mg	Cellulose, magnesium salts of fatty acids, hydroxypropylmethyl-cellulose, polyvinyl-polipyrrolidone, talc, titanium dioxide, iron oxides
2	Magnolia bark extract	30 capsules	Magnolol, honokiol	563 mg (3.26)	40 mg	10 mg	10 mg	Extract from the root of *Withania somnifera*, 112 mg (including 12.25% glycoside vitanolids); panthotenic acid, 6 mg; vitamin B6, 1.4 mg; folic acid, 200 μg; vitamin D, 50 μg; vitamin B12, 2.5 μg	Cellulose, hydroxypropylmethyl-cellulose, magnesium salts of fatty acids, titanium dioxide, brilliant blue FCF
3	Magnolia bark extract	60 capsules	Magnolol, honokiol	658 mg (1.36)	400 mg	No data	No data	No data	Brown rice flour, gelatine. Possible components: magnesium salts of fatty acids, silicon dioxide
4	Magnolia extract	30 capsules	Magnolol, honokiol	439 mg (1.32)	200 mg	No data	180 mg	No data	Rice flour, hydroxypropylmethyl-cellulose, magnesium salts of fatty acids, silicon dioxide
5	Magnolia extract	30 mL	Not detected	30 mL	No data	No data	No data	No data	Herbal extract from *Pueraria montana* radix (kudzu root), water, cellulose, xanthan, citric acid, potassium sorbate
6	Magnolia bark powder	160 capsules	Not detected	344 mg (4.40)	No data	2.5 mg	0.5 mg	No data	Gelatine, titanium dioxide

remaining six samples 1-6 (one tablet, four capsules, and one liquid), three independent specimens of each sample were processed separately in the following manner; sample 1 (tablet) was crushed in a mortar; samples 2-4 and 6 (capsules) were opened and emptied to the vessels; sample 5 (liquid) was collected in the aliquot of 1 mL. To each sample, understood as one tablet, content of one capsule or 1 mL liquid, 5-mL portions of methanol were added and the extraction was carried out with use of an ultrasonic bath (10 min ultrasonication at room temperature; 35 kHz; Sonorex RK 52 H, Bandelin Electronic, Berlin, Germany). After the extraction, samples were left for ca. 5 min for the solid matter to sediment, followed by filtration, first with a paper filter (75 $\pm$ 2 g m^{-2}) and then a syringe filter (Anatop 0.02 μm, Φ 10 mm). After this stage, sample 4 was ten-fold diluted with methanol. These single extracts were used to calculate the precision of the method and variability of the contents of neolignans. For a quantitative assay and confirmation of the manufacturers' declaration, the three single extracted items of each dietary supplement (samples 1-4) were dried (in a fume hood and subsequently in a desiccator), pooled and underwent further two tandem extractions with methanol in the above described way. After each extraction step, the contents of magnolol and honokiol in the supernatant were quantified by means of the newly developed TLC method. The results from three consecutive extraction rounds were summed up to 100% of magnolol and honokiol, respectively.

For the purpose of the HPTLC-EDA analysis, samples denoted as Mix 1 and Mix 2 were dissolved in methanol (1 mg mL^{-1}). Solid samples 1-4 and 6 (100 mg of the powdered tablet or capsule) were vortexed in 1 mL methanol for 1 min and ultrasonicated for 10 min. The same procedure was performed with the liquid sample 5, ten-fold diluted with methanol. The supernatants were applied for bioactivity tests. Sample 4 had to be ten-fold diluted with methanol.

2.3. The silica gel-based TLC system to separate and quantify magnolol and honokiol

Thin-layer chromatographic analyses were carried out with TLC silica gel 60 F_{254} plates. The chromatograms were developed with the mobile phase *n*-hexane – ethyl acetate – ethanol, 16:3:1 (v/v/v). Each sample was spotted using a calibrated 5-µL glass micropipette (CAMAG, Muttenz, Switzerland) onto the chromatographic plate 10 mm above the lower plate edge and each development was preceded by pre-saturation of the chromatographic chamber with the mobile phase for 10 min. The migration distance of mobile phase from the lower plate edge was 130 mm

that took 35 min. The development of the chromatograms was carried out in standard 20 cm x 20 cm flat-bottomed chromatographic chambers (CAMAG). After development, plates were dried in ambient air. In order to produce the calibration curves for magnolol and honokiol, both standards were spotted onto chromatographic plates in aliquots from 1 to 6 μL in 1-μL intervals. Each analysis was performed with four replications (n = 4). Linearity range, limit of detection (LOD) and limit of quantification (LOQ) were determined for both neolignans. The dietary supplements (samples 1-6), each analyzed as three different specimens, were spotted onto chromatographic plates in 1-μL aliquots, and these analyses were performed in triplicate (n = 3). Mix 1 and Mix 2 samples were spotted onto chromatographic plates in 5-μL aliquots and these analyses were also performed in triplicate (n = 3). Variability of the neolignans content of the tablets/capsules was also determined.

2.4. Densitometry

For quantification of magnolol and honokiol, the chromatograms were scanned with a densitometer model CD 60 equipped with Pro-Quant software compatible with Microsoft Windows operating system (Desaga, Wiesloch, Germany). Densitometric scans were performed in the absorbance mode at wavelength λ = 290 nm. Dimensions of the rectangular light beam were 0.1 mm × 1.0 mm.

2.5. HPTLC-EDA

Samples were applied onto the HPTLC layer by a TLC sampler (ATS3, CAMAG) as the 5-mm bands with 9-mm track distance and 8-mm distance from the lower plate edge. HPTLC separation was carried out with *n*-hexane – ethyl acetate – ethanol (16:3:1, v/v/v) in a saturated (10 min) 10 cm × 10 cm twin-trough chamber (CAMAG) to a migration distance of 70 mm. After development, the plates were dried in a cold air stream and documented by a digital camera (Cybershot DSC-HX60, Sony, Neu-Isenburg, Germany) under a UV lamp (CAMAG) and by a TLC Scanner 3 densitometer at 254 nm or 290 nm with slit dimension 5 mm x 0.2 mm (CAMAG). Thereafter, the plates were subjected to different biological assays.

Antibacterial activity was tested against *A. fischeri* and *B. subtilis* strains, applying the previously described HPTLC-direct bioautographic methods [37, 38]. Briefly, the freshly prepared and dried chromatograms were immersed into an appropriate cell suspension. Inhibitory activity, indicated by dark spots in the luminescent bioautograms (*A. fischeri*), was documented at an exposure time of 50 s using an iBright™ FL1000 Imaging System (Thermo Fisher Scientific, Budapest, Hungary), whereby the bioautograms were kept under a glass cover assuring sufficient humidity. The *B. subtilis* bioautograms were visualized after 2 h incubation (at 28 °C in 100% humidity), by immersion into an aqueous MTT solution (1 mg mL^{-1}). The inhibition zones were revealed as bright spots against a bluish background, which was documented by a Cybershot DSC-HX60 camera. Free radical scavenging activity was detected by immersion of the chromatogram into a methanolic DPPH$^{\cdot}$ solution (0.02%). Bright zones against a purple background indicated antioxidants. Documentation was performed with a Cybershot DSC-HX60 camera using the transmitted white light illumination.

2.6. HPTLC-MS

For the mass spectrometric experiments, the online combination of a binary pump (LC-20AB, Shimadzu, Kyoto, Japan), a TLC-MS Interface (CAMAG) using an oval elution head (4 mm × 2 mm) and a single quadrupole electrospray ionization mass spectrometer (LCMS-2020, Shimadzu) were utilized. The instrument control and data acquisition were performed using the LabSolutions 5.42v software (Shimadzu). The mass spectrometric conditions were as follows: the nebulizer gas (N_2) flow rate, 1.5 L min^{-1}; the drying gas (N_2) flow rate, 10 L min^{-1}; the interface temperature, 350 °C; the heat block temperature, 400 °C; the desolvation line temperature, 250 °C; the detector voltage 4.5 kV. The full mass scan spectra were recorded in the positive and negative ionization mode, in the *m/z* range of 150–800, with a scan speed of 682 amu/s. Zones of interest were eluted with methanol (gradient grade) at a flow rate of 0.2 mL min^{-1}.

2.7. Stability assessment by 2D-HPTLC

Undiluted sample 4 (1 µL) or mixture of standards (1 μg of each Mix 1 and Mix 2) was spotted onto the HPTLC layer with a microsyringe at a 10-mm distance from the lower plate edge and developed with *n*-hexane – ethyl acetate – ethanol (16:3:1, v/v/v). The dried plate was kept for 20 h in a box to protect from the light and developed again with a mobile phase of the same composition in the orthogonal direction. The dried plate was documented and then underwent the HPTLC-MS analysis.

3. Results and discussion

3.1. Quantification of magnolol and honokiol

In order to quantify the two neolignans magnolol and honokiol (Fig. 1) in the investigated dietary supplements (samples 1-6) and in the magnolol and honokiol mixtures (Mix 1 and Mix 2), first a relatively cheap TLC-densitometry method was developed. As mobile phase various mixtures of toluene, n-hexane, isopropyl acetate, ethyl acetate, ethanol, and methanol, were tried in the normal phase TLC (Fig. S-1). The separation of magnolol and honokiol was achieved on the silica gel plates after manual spotwise sample application using *n*-hexane – ethyl acetate – ethanol (16:3:1, v/v/v) mobile phase and 130 mm as the development distance (Fig. 2). To elaborate the calibration curves, solutions of the magnolol and honokiol standards were spotted onto the chromatographic plates in aliquots from 1 to 6 μL, in 1-μL intervals. After development, chromatograms were densitometrically scanned in absorbance mode at wavelength λ = 290 nm, the common absorbance maximum of the two neolignans (Fig. 3). Quantification was performed based on the chromatographic peak height values. Numerical values of the detection and quantification limits (LOD and LOQ, respectively) were calculated using the following formulas: LOD = 3.3 × SD/*a*, and LOQ = 10 × SD/*a*, where SD is the standard deviation of peak height, and *a* is the slope of the calibration curve ($y = ax + b$). Each chromatographic analysis was repeated four times (n = 4). Respective retardation factors (hR_F), calibration curve equations, determination coefficient values (R^2), linearity range, and LOD and LOQ values calculated for magnolol and honokiol are given in Table 2. The obtained numerical results

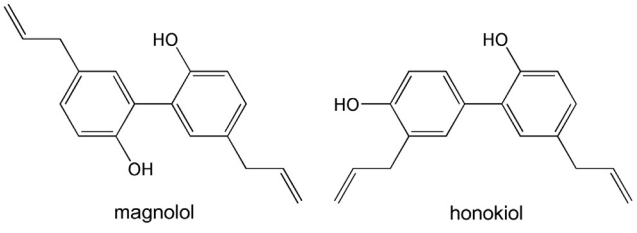

Fig. 1. Chemical structures of magnolol and honokiol.

Table 2
Retardation factor (hR_F) values, linear regressions, determination coefficients (R^2), the LOD and LOQ values, and the linearity range obtained for magnolol and honokiol in the employed chromatographic system (silica gel TLC plates, *n*-hexane - ethyl acetate - ethanol, 16:3:1 (v/v/v)); n = 4.

Standard compound	hR_F (±1)	Linear regression	Determination coefficient (R^2)	LOD [μg spot^{-1}] (RSD (%))	LOQ [μg spot^{-1}] (RSD (%))	Linearity range [μg spot^{-1}]
Magnolol	39	y = 321.3x + 52.6	0.99	0.09 (11.4)	0.28 (11.4)	0.09 – 0.58
Honokiol	34	y = 433.1x + 80.2	0.98	0.07 (8.1)	0.20 (8.1)	0.07 – 0.57

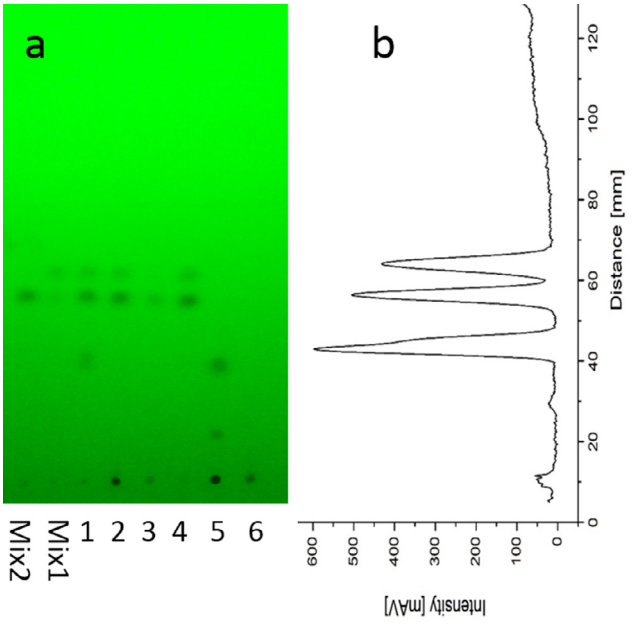

Fig. 2. Normal-phase TLC chromatogram (a) of dietary supplements (1-6: samples 1-6, respectively; 1 µL of each) and mixtures of standards Mix 1 and Mix 2 (5 µL of each) at UV 254 nm after the development with *n*-hexane – ethyl acetate – ethanol (16:3:1, v/v) to the 13 cm distance and densitogram (b) of sample 1 recorded at 290 nm.

Table 3
Precision of the developed method assessed in terms of the relative standard deviation (% RSD) for the contents of magnolol and honokiol in sample 2 expressed in mg g^{-1} sample. Six different capsules (a)–(f) of sample 2 were tested and the respective extracts were chromatographed on two different chromatographic plates, with each sample being spotted onto the chromatographic plate in triplicate (n = 3).

Sample 2	Magnolol			Honokiol		
	hR_F (±0.1)	mg g^{-1}	RSD [%]	hR_F (±0.1)	mg g^{-1}	RSD [%]
(a)	38	12.1	3.1	34	13.1	4.2
(b)	37	11.6	1.8	32	12.5	1.5
(c)	38	11.8	2.6	33	12.8	3.2
(d)	38	12.2	2.1	33	12.7	2.0
(e)	38	13.6	1.2	32	13.8	0.9
(f)	39	11.9	2.6	33	12.1	2.3

demonstrated very good detectability and good quantification levels, with the LOD and LOQ values for magnolol equal to 0.09 and 0.28 μg spot^{-1}, respectively, and for honokiol equal to 0.07 and 0.20 μg spot^{-1}, respectively.

Precision of the developed quantification method was assessed in terms of the relative standard deviation (% RSD), using individual extracts of sample no. 2 as an example. To this effect, six different capsules (a)–(f) of sample 2 were tested and the respective extracts were chromatographed on two different chromatographic plates, with each sample being spotted onto the plate in triplicate (n = 3). The results obtained are given in Table 3. The

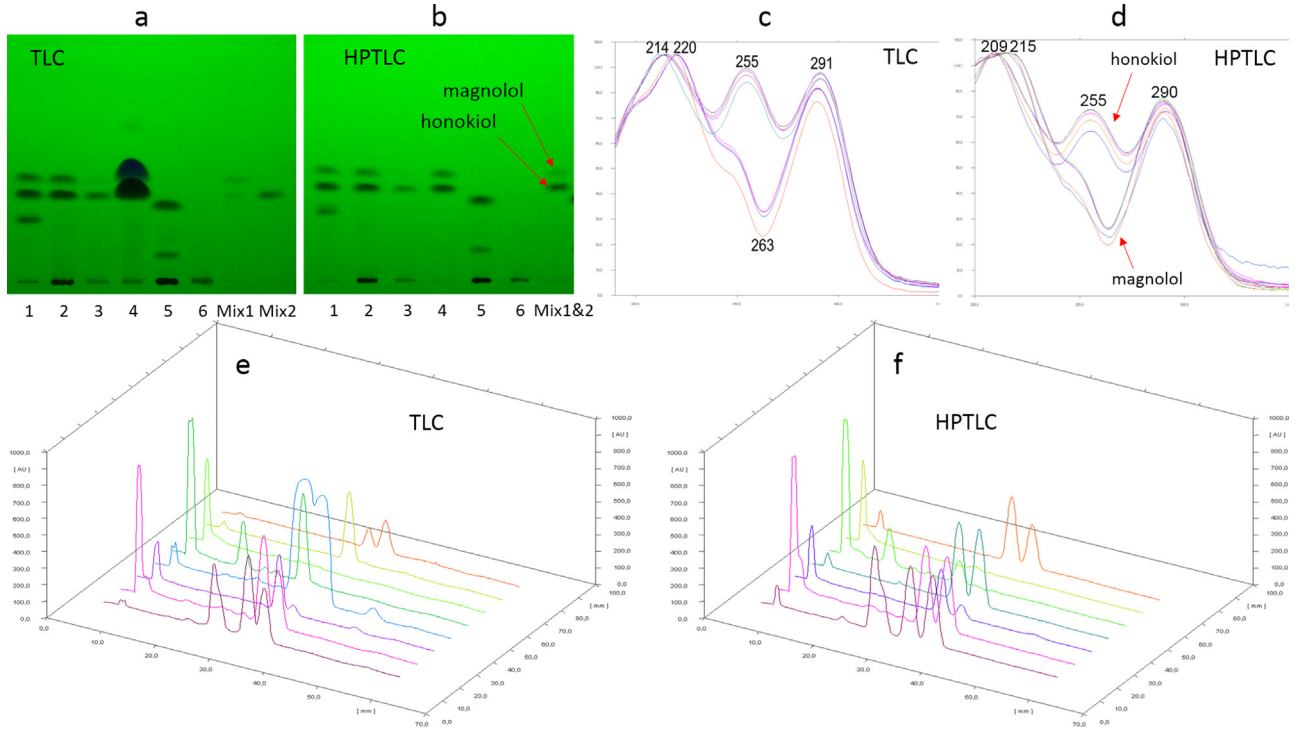

Fig. 3. Normal-phase TLC (a) and HPTLC (b) chromatograms of dietary supplements (1-6: samples 1-6, respectively; sample 4 in TLC before the ten-fold dilution) and mixtures of standards Mix 1 and Mix 2 at UV 254 nm after the development with *n*-hexane – ethyl acetate – ethanol (16:3:1, v/v) to the 7 cm distance, and UV spectra (c,d) recorded in the zones of interest (mixtures of standards and samples) and densitograms (e,f) recorded at 254 nm (e) and 290 nm (f) after the TLC (c,e) and HPTLC (d,f) development. The applied sample volumes were 0.5 µL and 0.2 µL into TLC and HPTLC layers, respectively.

Table 4
The contents of magnolol and honokiol in mg g^{-1} sample for each investigated dietary supplement (samples 1-6) and variability of the contents expressed as the relative standard deviation (% RSD). The results hold for two different sets of extracts from samples 1-6, each sample spotted onto the chromatographic plate in triplicate (n = 3).

Sample no.	Magnolol			Honokiol		
	hR_F (±1)	mg g^{-1}	RSD [%]	hR_F (±1)	mg g^{-1}	RSD [%]
1	39	9.9	14.1	34	8.3	12.4
2	38	15.1	8.4	33	13.0	7.6
3	39	0.9	2.8	33	4.5	5.2
4	39	112.4	0.3	32	82.8	1.0
5	–		–	–		–
6	–		–	–		–

obtained quantification data fall within a relatively narrow % RSD range (equal to 1.2–3.1 % RSD for magnolol and 0.9–4.2 % RSD for honokiol), showing satisfactory precision of the developed analytical approach.

Mix 1 and Mix 2 were declared by the donator as mixtures of magnolol and honokiol alone (without a matrix). From our analytical results it came out that the content of magnolol in Mix 1 was equal to 910.6 ± 75.7 mg g^{-1} (n=3), i.e., to ca. 91.1 weight % of the sample, and the content of honokiol was below the LOQ value. As Mix 1 consists of magnolol and honokiol alone, it can be concluded that the content of honokiol equals to ca. 8.9 weight % in this sample. With Mix 2, the content of honokiol was found as equal to 633.2 ± 22.8 mg g^{-1} (n=3), i.e., to ca. 63.3 weight % of the sample, and magnolol was not detected. Hence, a conclusion was drawn that the content of magnolol in Mix 2 equals to ca. 36.7 weight % of the sample.

Variability of the contents of magnolol and honokiol per each investigated dietary supplement (samples 1-6) was assessed in terms of the relative standard deviation (% RSD), based on two different sets of single extracts obtained from two different specimens of each sample spotted onto the chromatographic plate in triplicate (n = 3). The results obtained are presented in Table 4. The highest variability of the contents was observed with sample 1, which was a coated tablet (14.1 and 12.4% RSD for magnolol and honokiol, respectively). Lower variability was observed with samples 2 and 3, which were capsules, and the lowest variability was observed with sample 4, which was also a capsule (0.3 and 1% RSD for magnolol and honokiol, respectively). In samples 5 and 6, neither magnolol nor honokiol was detected, either due to their absence in these two samples, or because of their contents falling below the established LOD values. All the obtained numerical results are acceptable and the observed differences seem justified by different preparation technologies with tablets and capsules. In fact, one can expect the highest variance in amount of the targeted compound among coated tablets (e.g., due to an uneven thickness of the coating layer) and lower variances among the capsules.

To determine the extractable amount of magnolol and honokiol contained in the samples 1-4, a quantitative assay was performed using repeated exhaustive extraction. Each sample underwent three consecutive extraction rounds and after each extraction, magnolol and honokiol was quantified by means of TLC. The quantification yields obtained from the consecutive extraction rounds were summed up to 100% and the results (valid per 1 gram of the tablet or capsule) are given in Table 5. Among the dietary supplements considered, the highest contents of magnolol and honokiol were found in sample 4 (114.7 mg g^{-1} magnolol and 84.9 mg g^{-1} honokiol). The lowest detectable amount of magnolol and honokiol were found in sample 3 (0.9 mg g^{-1} magnolol and 4.9 mg g^{-1} honokiol). The contents were re-calculated per weight of a single tablet or capsule (as given in Table 1), and our results were compared with the manufacturers' declarations. The most accurate data were provided by the manufacturer of sample 2 (capsule weight, 563 mg; the declared contents of magnolol and honokiol, 10 mg each). Our quantification revealed 9.6 mg magnolol and 8.2 mg honokiol per capsule, which remains in a fairly good agreement with the declaration. The weight of sample 1 was 604 mg, no information was available regarding the content of magnolol and the amount of honokiol was declared as 21 mg per tablet. Our assay revealed 8.2 mg magnolol and 6.7 mg honokiol per tablet (i.e., by one third less honokiol than declared). Manufacturer of sample 3 did not provide any quantitative information regarding the contents of magnolol or honokiol in its capsule (658 mg), and our assay revealed 0.9 mg magnolol and 4.9 mg honokiol. For sample 4 (capsule, 439 mg), the manufacturer provided no information regarding the content of magnolol, but the declared amount of honokiol was 180 mg. From our measurement it came out that sample 4 contains 50.4 mg magnolol and 37.3 mg honokiol per capsule (i.e., ca. 20 % of the declared honokiol content). Summing up, the quantitative assay of magnolol and honokiol performed with use of the proposed thin-layer chromatographic method demonstrates its practical usefulness for quantification and quality control of the magnolol and honokiol containing dietary supplements and similar preparations from the group of foodstuffs.

Table 5
Quantitative assay of magnolol and honokiol for samples 1-4 measured per one gram of samples 1-4 in three consecutive steps of exhaustive extraction (nd holds for not detected).

Sample no.	Exhaustive extraction no.	Magnolol		Honokiol	
		[mg g^{-1}]	[%]	[mg g^{-1}]	[%]
1	1	10.5 ± 0.2 (n=3)	76.7	8.4 ± 0.3 (n=3)	75.9
	2	3.2	23.3	2.7	24.1
	3	nd	nd	nd	nd
	Σ	13.7	100	11.1	100
2	1	15.6 ± 1.5 (n=3)	91.5	13.1 ± 1.0 (n=3)	90.6
	2	1.4	8.6	1.4	9.4
	3	nd	nd	nd	nd
	Σ	17.1	100	14.5	100
3	1	1.0 ± 0.1 (n=3)	100	4.9 ± 0.3 (n=3)	100
	2	nd	nd	nd	nd
	3	nd	nd	nd	nd
	Σ	1.0	100	4.9	100
4	1	108.0 ± 7.6 (n=3)	94.13	79.4 ± 5.4 (n=3)	93.6
	2	6.7	5.9	5.5	6.4
	3	nd	nd	nd	nd
	Σ	114.7	100	84.9	100

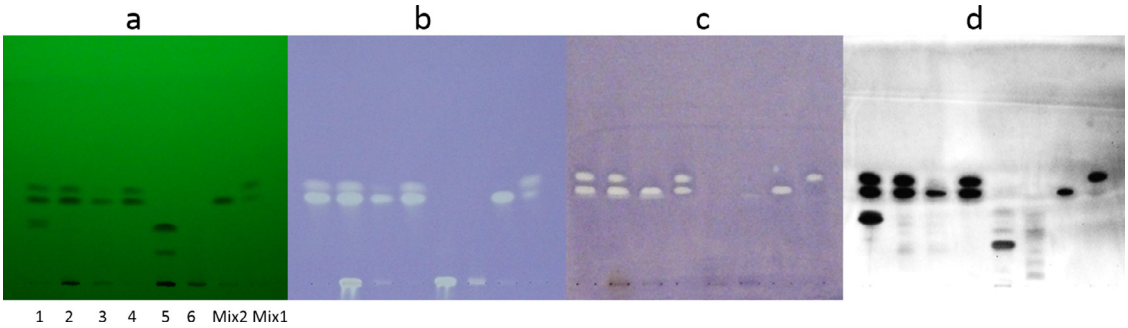

Fig. 4. HPTLC chromatograms of bioactive components in dietary supplements (1-6: samples 1-6, respectively; 0.2 µL of each) and mixtures of standards Mix 1 and Mix 2 (0.5 µL of each) at UV 254 nm (a), and after DPPH· test (b), and *B. subtilis* (c) and luminescent *A. fischeri* (d) bioassays.

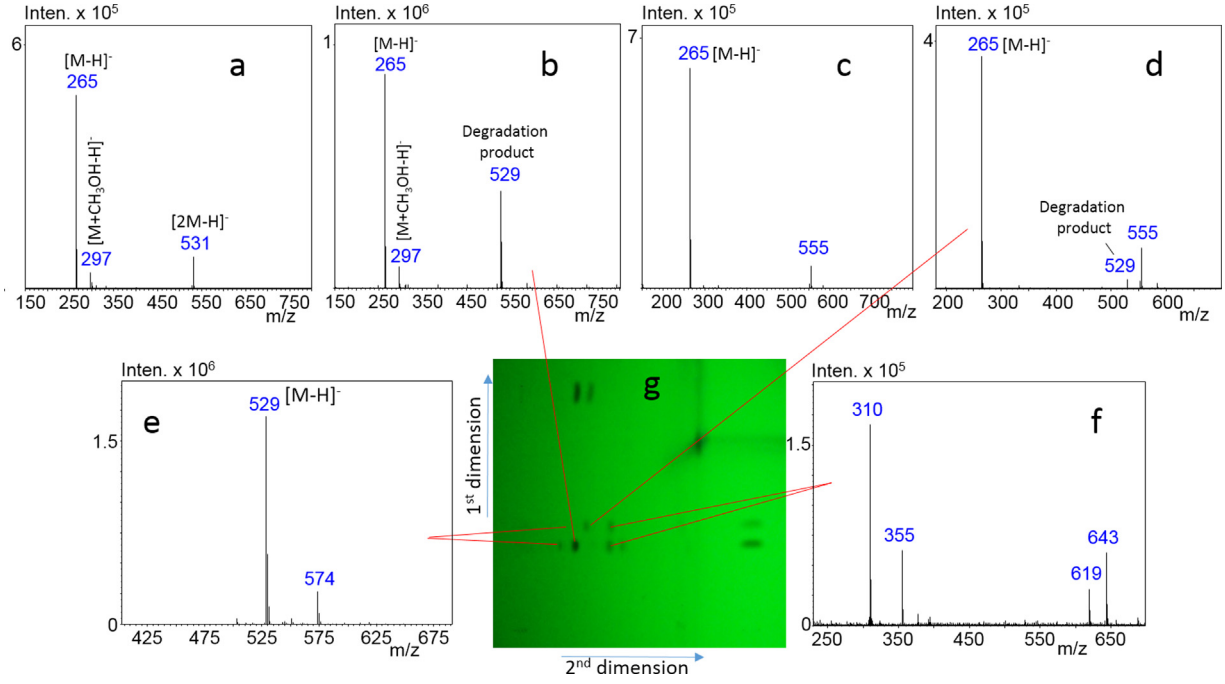

Fig. 5. Direct MS analysis of Mix 2 (a) and Mix 1 (c) deposited on the aluminium foil and the HPTLC-ESI⁻-MS full scan spectra of the honokiol (b), magnolol (d) and their degradation products (e,f) after the 2D-HPTLC separation of the mixtures of standards (1 µg of each). Chromatogram (g, at UV 254 nm) was developed with *n*-hexane – ethyl acetate – ethanol (16:3:1, v/v) in both directions using the 20-h break. Mass spectra were obtained via the elution head-based TLC-MS Interface and background subtraction.

3.2. HPTLC-EDA

The relatively cheap silica gel-based TLC method comprising spot-wise sample application by a calibrated capillary was intended to be improved by an instrumental band-wise sample application. A reduced initial band broadening allowed the TLC separation of magnolol and honokiol (R_s = 0.9) in a shorter development time (12 min) and at a shorter distance (7 cm) (Fig. 3 a and e). Peak purity was established by recording the UV (Fig. 3 c) and mass spectra (not shown) of the zones of interest. Even better separation was achieved with the HPTLC silica gel adsorbent (Fig. 3 b, d and f), resulting in a higher resolution (R_s = 1.2) and slightly higher hR_F values (46.1 and 39.5 for magnolol and honokiol, respectively). Bioprofiles of the dietary supplements were determined by HPTLC combined with an antioxidant (DPPH·) and antibacterial assays. Magnolol and honokiol displayed antioxidant activity and inhibited the Gram positive *Bacillus subtilis* and the Gram negative naturally luminescent *Aliivibrio fischeri* bacterial strains (Fig. 4). Both compounds could be detected by all bioactivity assays in samples 1, 2 and 4. In sample 3, only honokiol was contained in the minimum effective amount, while in samples 5 and 6 neither magnolol, nor honokiol was observed. Apart from chromatographic zones of the two isomeric biphenyl neolignans, zones with antioxidant activity appeared at the application bands of samples 2, 5 and 6. Interestingly, *B. subtilis* was not sensitive to any sample component other than magnolol and honokiol. Antioxidant activity of these neolignans [12, 13, 22] and their antibacterial effect against *B. subtilis* [17] have been recognized earlier. However, to the best of our knowledge, antibacterial effect of magnolol and honokiol against *A. fischeri* has not yet been reported.

Additional inhibition zones against *A. fischeri* were detected in the tracks of samples 1 and 5. In sample 1, two additional and incompletely separated active zones at hR_F 29 and 30.3 were found. Densitometric assessment of the peak shoulder also confirmed the presence of at least two compounds. The oval elution head of the TLC-MS interface allowed collective elution of these two zones and the mass spectrometric evaluation resulted in detection of characteristic mass signals at *m/z* 308, *m/z* 340 and *m/z* 593 in the positive ionization mode, which were assigned as the sodium adduct $[M+Na]^+$, the sodium and methanol adduct $[M+CH_3OH+Na]^+$ and the sodium adduct of the dimer $[2M+Na]^+$ (Fig. S-2a). The protonated molecular ion at *m/z* 286 was also detected, with a lower intensity though. These signals were tentatively ascribed to the geometric isomers of the alkaloid piperine, which appears as a de-

clared component of sample 1. *A. fischeri* also proved sensitive to a component of sample 5 at hR_F 17.5. The compound present in this chromatographic zone was characterized by mass spectrometry. In the negative ionization mode the deprotonated molecule $[M-H]^-$ at m/z 253 (Fig. S-2b) and in the positive ionization mode the sodium adduct $[M+Na]^+$, the sodium and methanol adduct $[M+CH_3OH+Na]^+$ and the sodium adduct of the dimer $[2M+Na]^+$ were recorded at *m/z* 277, *m/z* 309 and *m/z* 531 (Fig. S-2c), respectively. Based on the data taken from the literature [39, 40], this compound was tentatively identified as the isoflavone daidzein that could originate from the sample ingredient, kudzu root. Antibacterial effect of both piperine and daidzein against Gram positive and Gram negative strains has previously been reported [41, 42]. In our study though, concentrations of piperine and daidzein have proved effective in the Gram negative *A. fischeri* assay only.

3.3. Stability assessment by 2D-HPTLC

Stability of magnolol and honokiol on the silica gel adsorbent was assessed in the two-dimensional (2D) HPTLC mode using the 20-h break between the two orthogonal development runs with mobile phase of the same composition. After the 2nd development run, derivatives of neolignans were detected below and above their chromatographic zones (Fig. 5). Mass spectra of neolignans recorded upon their elution from the adsorbent (Fig. 5 b and d) were very similar to those obtained by eluting Mix 1 and Mix 2 deposited on an aluminium foil (Fig. 5 a and c). The only characteristic difference was a new signal that appeared on silica gel at m/z 529 in the negative ionization mode. It probably was the dimer of honokiol with monoisotopic mass of 530 (the component of *Magnolia officinalis* root [43]), which was formed from honokiol in the course of the chromatographic process. So far, the dimer of magnolol has not yet been described as a component of the magnolia bark, but it was synthesized in the laboratory only [44]. Based on the results originating from the 2D HPTLC experiment, compounds with the mass signal at m/z 529 might have been formed from both magnolol and/or honokiol (Fig. 5 e). Similarly, the respective degradation products with the hR_F values higher than those of the neolignans gave very similar mass spectra (Fig. 5 f). Fortunately, neither magnolol nor honokiol degrades on the silica gel adsorbent within 4 h storage period (Fig. S-3), so that there is enough time to document the chromatograms. Based on these observations, the UV densitometry and the bioactivity assays were performed immediately after the development and drying of the chromatograms.

Conclusions

The TLC-UV method was shown as an efficient and reliable tool for quantitative determination of the two magnolia neolignans, magnolol and honokiol, in dietary supplements. The developed method provided very good detectability, good quantification levels and satisfactory precision. The obtained quantitative results clearly confirmed the necessity of stricter quality control of the dietary supplements to exclude products with doubtful constitution and doubtful physiological effects from the market. The HPTLC-EDA/MS approach was found to be useful for the high-throughput bioprofiling of the dietary supplements to screen and identify bioactive ingredients. 2D-HPTLC-MS was shown as a suitable and easy-to-perform approach for stability assessment of the analytes of interest.

Credit authorship contribution statement

Eliza Łata & Agnieszka Fulczyk: Quantitative TLC Experiments, Data Analysis, Writing - Original Draft. **Péter G. Ott**: Methodology, Resources, Writing - Review & Editing. **Teresa Kowalska**: Conceptualization, Resources, Writing - Original Draft, Review & Editing. **Mieczysław Sajewicz**: Conceptualization, Resources, Writing - Review & Editing. **Ágnes M. Móricz**: Conceptualization, Methodology, Resources, HPTLC Experiments, Data Analysis, Writing - Original Draft, Review & Editing.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461230.

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